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(54) Title: BACTERIAL DETECTION BY PHAGE TRANSDUCTION OF DETECTABLE PHENOTYPE

(57) Abstract

Viable bacteria may be detected in biological samples by exposing bacterial cultures obtained from the samples to transdu-
cing particles having a known host range. Such transducing particles carry a heterologous gene capable of altering the phenotype
of the bacteria in a readily detectable manner. For example, the transducing particles may carry an ice nucleation gene and the
alteration of phenotype may be detected using an ice nucleation assay. By employing a panel of phage, unknown bacteria may be
typed based on the pattern of reactivity observed.

BACTERIAL DETECTION BY PHAGE
TRANSDUCTION OF DETECTABLE PHENOTYPE

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the detection and identification of bacteria in biological samples. More particularly, the present invention relates to the screening of biological samples with bacteriophage capable of specifically infecting cells of interest and transforming the infected cells to a detectable phenotype.

The detection and identification of bacterial species and strains is of interest under a variety of circumstances. For example, there is a need to be able to rapidly screen food, water, and other comestibles for contamination with pathogenic bacteria. The detection of bacteria in patient samples is similarly necessary in the treatment of numerous infectious diseases. In the latter case, it is frequently desirable to be able to specifically type the bacteria and would be further desirable to screen the bacteria for sensitivity to various bacteriocides.

Heretofore, various techniques have been used for bacterial identification, including serotyping, nutritional screening, and phage typing. Serotyping utilizes a panel of antibodies capable of binding to distinct cell surface antigens on target bacteria. Based on the observed pattern of binding, the species and strain of the bacteria may be determined. Nutritional screening relies on variations in the metabolic requirements of different types of bacteria. By growing (r attempting t grow) the bacteria on well-defined

media, the bacteria may be classified based on those substances which are necessary for growth and those substances which inhibit growth.

Of particular interest to the present invention, bacteriophage have been used to type bacterial cultures based on the limited host range of different phage. By attempting to infect aliquots of a pure culture of unknown bacteria with a panel of different phage, the bacterial cell type can be determined.

While such phage typing is a highly accurate and determinative procedure for identifying bacterial type, it suffers from being both time consuming and labor intensive. Bacteria in a sample must first be grown out so that pure cultures may be isolated. Individual colonies of the pure cultures must then be grown and subsequently divided into aliquots which are exposed to the different phage in the panel. After exposure, conventional plaque assays are run to determine the infectivity of the various phage. The entire procedure takes from 24 to 48 hours, or longer, and requires highly trained personnel for execution. Because of the lengthy procedure, and the need to identify plaques in the bacterial colonies, the procedure is not amenable to automation. Moreover, because of the time required, the procedure is less than ideal for determining the nature of a patient's infection prior to therapy.

Disabled bacteria, such as those debilitated by cooking or partial heat sterilization, are a major detection problem in many food processing situations. Such disabled bacteria frequently remain viable (and thus potentially pathogenic) yet are sufficiently weakened so that detection by conventional assay protocols may require a non-selective recovery step (pre-enrichment) followed by a selective enrichment step to allow growth of the targeted bacteria while growth of c mpeting rganisms is inhibited. Such additional steps can

significantly add to the time required to perform the assay.

Detection of particular bacteria in open environments, such as air, water, and soil, can also be problematic. Because of the wide variety of species that may be present, it will often be difficult to distinguish a particular species of interest.

In view of the above, it would be desirable to provide improved phage screening assays for detecting and identifying bacterial cells in biological samples. In particular, it would be desirable if such assays could be performed in a relatively short period of time and that the assay protocols be sufficiently simple to be performed by semi-skilled personnel. Preferably, the assays will be performed on mixed cultures, with a minimum number of steps, and result in a detectable event which is easily observed and amenable to automated reading. It would be further desirable if the assays were able to detect disabled bacteria which might otherwise require a pre-enrichment step and a selective enrichment step for detection. In addition, it would be desirable to be able to rapidly and conveniently detect particular bacterial cells in open environments, such as air, water, and soil.

2.   Description of the Background Art

The use of bacteriophage in characterizing the surface of bacterial cells is discussed in Makela, *Enterobacterial Surface Antigens: Methods for Molecular Characterization*, Korhonen et al., eds., Elsevier Science Publishing, Amsterdam, pp. 155-178 (1985), where conventional plaque assays are employed to determine infectivity of particular phage. The molecular genetics of bacteriophage P22 is discussed in Susskind and Botstein (1978) Microbiol. Rev. 42:385-413. The ability of P22 to act as a transduction vector is described in Watanabe et al. (1972) Virol. 50:874-882 and Orbach and Jackson (1982) J. Bacteriol. 149:985-994. The use of P22

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to selectively transduc recombinant plasmids with
integrated pac sequences is described in Schmidt and
Schmieger (1984) Mol. Gen. Genet. 196:123-128 and Vogel
and Schmieger (1986) Mol. Gen. Genet. 205:563-567.
Foreign genes are inserted into bacteriophage L (related
to P22) by transposon mutagenesis, as described in
Spanova and Karlovsky (1986) Folia Microbiol. 31:353-363.
The use of λ phage as a cloning vector is described in
Young and Davis (1983) Proc. Natl. Acad. Sci. USA
80:1194-1198, where unknown gene products may be detected
by antibody probes. The use of M13 phage as a cloning
vector is described in Vieira and Messing (1987) Meths.
Enz. 153:3-11.

Bacteria may be detected in biological samples
by a number of techniques, including selective media,
immunoassays, and nucleic acid probes. Particular
methods for detecting *Salmonella* are described in U.S.
Patent No. 4,689,295. A phage-based test for detecting
Salmonella in food is described in ASM News (1987)
53:542. The test uses phage to mediate the adsorption of
the target *Salmonella* on a surface.

The ability to nucleate ice formation has been
reported to be encoded by a single gene in several ice
nucleation-positive (Ina+) bacteria, and this ability can
be transferred to E. coli by transformation with a
plasmid carrying the ice nucleation gene. See, U.S.
Patent No. 4,464,473; Orser et al., *Molecular Genetics of
the Bacterial-Plant Interaction* (A. Puhler, ed.),
Elsevier/North Holland Biomedical, pp. 353-361 (1983);
Green et al. (1985) Nature 317:645-648; and Corotto et
al. (1986) EMBO J. 5:231-236. Sequence information for
an ice nucleation gene in *P. syringae* (gene *inaZ*) has
been reported; Green et al. (1985) *id*. The corresponding
protein is of approximate molecular weight 1.2×10^5.
Information concerning the identification and
purification of this protein is reported in Wolber et al.
(1986) Proc. Natl. Acad. Sci. USA 83:7256-7260. Sequence

information for an ice nucleation gene in *P. fluoresc ns*
(gene *inaW*) has also been rep rted. See, Warren t al.
(1986) Nuc. Acids. Res. 14:8047-8060.

The droplet freezing assay is a known method of
testing for the presence of whole cell ice nucleating
bacteria and cell-free nuclei. The method consists of
laying out an array of N droplets of volume V (usually
0.01 ml) on a nucleus-free surface, cooling to
temperature T (less than 0°C) and scoring N_f, the number
of droplets frozen. The number of nuclei/ml is then
calculated by the following formula: nuclei/ml=(1/V) $\log_e$
[N/(N-N_f)]. G. Vali (1971) J. Atmos. Sci. 28:402-409.

SUMMARY OF THE INVENTION

According to the present invention, methods and
compositions are provided for detecting and identifying
viable bacteria in biological samples. For these
purposes, the term viable bacteria includes all bacteria
capable of expressing genes (transiently or otherwise).
The compositions comprise bacteriophage particles capable
of infecting a known host range of bacteria and
transducing such bacteria to a readily detectable
phenotype, preferably an ice nucleation phenotype. The
method of the present invention is suitable for detecting
most types of bacteria, with the limitation that the
target bacteria must be susceptible to bacteriophage
infection and the wild-type bacteria cannot itself
produce the detectable phenotype. Biological samples of
interest may be obtained from virtually any source
capable of supporting or preserving bacteria in a viable
condition, including patient specimens, water, dairy
products, meat products, and the like. Such samples will
often contain mixed or impure populations of bacteria,
possibly but not necessarily containing a target bacteria
of interest. A preferred method of the present inventi n
is the detecti n of *Salmonella*, particularly in f od.

The method of the pr sent invention may be used
either f r screening samples for the presence of bacteria

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or for typing bacteria of an unknown strain or species.
For scr ening, only a single bacteriophage having a
relativ ly broad host range will be necessary. The
biological sample is incubated under conditions suitable
for promoting the growth of bacteria, and there is no
need to provide a pure culture. Such screening
procedures are very rapid and simple and are particularly
useful in identifying contaminated samples, such as food
or water samples. Bacterial typing, in contrast,
utilizes a panel of bacteriophage having distinct or
overlapping host ranges. Each of the bacteriophage is
tested against a culture (pure or otherwise) of the
bacteria, typically by adding various members of the
phage panel to aliquots of the culture to be tested. The
type of bacteria is then determined based on the pattern
of reactivity of the individual bacteriophage. Although
typing may involve the use of a pure culture of the
bacteria, the method of the present invention has the
advantage that the detection of phage infectivity is very
rapid, greatly shortening the overall time required for
the assay. Moreover, the detectable phenotype, such as
ice nucleation, may readily be detected by automated
systems, reducing the labor required for the assay.

The method of the invention takes advantage of
the specificity of bacteriophage for particular bacteria.
This specificity is retained when the bacteriophage are
modified to contain a marker gene (see Example 5). As a
consequence, detection via the assay of the invention can
be accomplished on a specific basis, irrespective of the
presence of other (non-target bacteria). Of course, it
'is possible (although unlikely) that the modified
bacteriophage of the present invention may have some
change in their host range specificity when compared to
the wild type phage from which they have derived. Such
variati n will n t be a pr blem so long as the
bacteri phage retains specificity for the bacteria f

interest and is free from specificity for other bacteria which might be present in a particular sample.

An important advantage of the method of the invention is that it may be used in assays involving bacterial cells which are viable but disabled (physiologically compromised) in some fashion, e.g., by exposure to injurious or debilitating treatments such as heat, cold or desiccation. Such disablement commonly results during industrial processing, e.g., processing of food-borne bacterial contaminants. The assay of the invention is capable of detecting disabled cells (Example 4, involving heat disabled *Salmonella* cells), including disabled cells in a mixture of healthy and disabled cells. This provides an important time-saving feature in that it minimizes or eliminates the need for a pre-enrichment step and a selective enrichment step. Such steps are commonly used in other bacterial assays as a means to allow the target cells to multiply to a detectable level while the populations of other organisms are kept in check. See Andrews, *Injured Index and Pathogenic Bacteria*, Boca Raton, Florida, pp. 56-113 (1989). Because debilitated cells may be unable to survive the rigors of selective enrichment, a prior nonselective "pre-enrichment" step is often employed to allow the weakened cells to stabilize. The FDA *Salmonella* isolation protocol recommends a minimum of 22 hours for each of the two steps (pre-enrichment and selective enrichment); Andrews et al., *Bacteriological Analytical Manual*, 5th Edition, Food and Drug Administration, District of Columbia, Chapter VI, 1-29 (1978). The method of the invention thus offers considerable time saving advantages when applied to systems having disabled cells.

In the preferr d embodiment, the transducing phage carries an ice nucleati n gene. The sample is first incubated with the phage under conditions which promote attachment of the phage to the cell, typically at

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a temperature in the range from about 35°C to 40°C
without agitation for a time in the range for about 15 to
120 minutes. Thereafter, the sample is incubated in a
suspension under conditions which promote development of
the ice nucleation phenotype, typically at a temperature
in the range from about 20°C to 25°C for a period in the
range from about 30 minutes to 2 hours. Bacteria
transformed to the ice nucleation phenotype are detected
by a conventional cryoassay, typically by dividing the
suspension to be tested into droplets having volumes
below about 10 μl and exposing the droplets to a
temperature in the range from about -3°C to -12°C, more
usually in the range from about -8°C to- 10°C. The
formation of ice nuclei in such a temperature range
indicates the presence of ice nucleation sites on the
cell surfaces of the bacteria.

Description of the Specific Embodiments

The present invention utilizes modified
bacteriophage, referred to hereinafter as transducing
particles, in order to detect or identify bacterial cells
in biological samples. As used herein, the term
transducing particles shall also include component parts
of a modified bacteriophage which component parts, when
mixed together under proper conditions, will combine to
form the modified bacteriophage. The biological samples
may be virtually any substance or medium capable of
supporting bacterial growth or otherwise suspending
bacterial cells in a viable state. Biological samples of
particular interest to the present invention include
water, soil, food samples, such as meat products and
dairy products which are particularly susceptible to
bacterial contamination, patient samples, such as blood,
plasma, serum, sputum, semen, saliva, lavage, feces, cell
culture, and the like.

The range of bacterial cells t be detected is
limited nly by host ranges of availabl bacteriophage.
Of particular interest are pathogenic bacteria which are

capable of contaminating food and water supplies and are
responsible for causing diseases in animals and man.
Such pathogenic bacteria will usually be gram-negative,
although the detection and identification of gram-

5   positive bacteria is also a part of the present
invention. A representative list of bacterial hosts of
particular interest together with the diseases caused by
such hosts and the bacteriophage capable of infecting
such hosts is presented in Table 1.

TABLE 1

Bacteria	Gram type	Diseases Caused	Bacteriophage
Bordetella pertussis	negative	whooping cough	See, N.A. Pereversev et al. (1981) Zh. Mikrobiol. 5:54-57
Brucella abortus	negative	brucellosis	TB; 212; 371
Mycobacterium tuberculosis	---	tuberculosis	LG; DSGA
Salmonella typhi	negative	typhoid fever	163; 175; ViI; ViVI; 8; 23; 25; 46; 175
Salmonella typhimurium	negative	gastroenteritis; septicemia	L; P22; 102
Salmonella schottmulleri	negative	gastroenteritis; septicemia	31; 102
Salmonella cholerae suis	negative	gastroenteritis; septicemia	102
Serratia marcescens	negative	wound infections	S24VA
Shigella dysenteriae	negative	bacterial dysentery	ϕ80; P2; 2; 37
Staphylococcus aureus	positive	toxic shock, infections	K; P1; P14; UC18; 15; 17; 29; 42D; 47; 52; 53; 79; 80; 81; 83A; 92

5	*Streptococcus pyog nes*	positive	streptococcal infections	φX240; 1A; 1B; 12/12; 113; 120; 124
	Vibrio cholerae	negative	cholera	138; 145; 149; 163
	Yersinia pestis	negative	plague	R; Y; P1
10	*Listeria monocytogenes*	positive	meningitis, abcess	243
	Ps udomonas a ruginosa	negative	wound and burn infection	B3; pp.7
15	*Escherichia coli*	negative	urinary infection	P1; /; T4
20	*Klebsiella pneumoniae*	negative	respiratory, urinary infection	60; 92

The transducing particles of the present invention are obtained by modifying a naturally-occurring bacteriophage to carry a gene capable of transforming the target bacteria to an easily recognizable phenotype,

5   referred to hereinafter as the primary marker gene. The transducing particle must be capable of specifically introducing the primary marker gene into the target bacterial host in such a way that the bacterial host can express the gene function in a detectable manner. A

10   large number of bacteriophage exist and may be selected for modification based on the desired host range and the ability of the bacteriophage to carry and transduce the gene of interest. In particular, the bacteriophage must be large enough to accomodate the primary marker gene,

15   associated promoter region, and any other DNA regions which may be included. Modified bacteriophage of the present invention will usually retain the normal host range specificity of the unmodified bacteriophage, although some alteration in specificity would be

20   acceptable so long as it does not affect the ability to identify the selected target bacteria.

The bacteriophage to be modified may be temperate or virulent, preferably being temperate in order to provide prolonged expression of the primary

25   marker gene in the target bacteria. Alternatively, modification of the bacteriophage may result in a defective transducing particle which is capable of introducing the marker gene into a target bacterial host, but which is incapable of achieving lytic or lysogenic

30   infection. In the latter case, the primary marker gene may be part of a plasmid or other self-replicating episomal unit which will be sustained and expressed in the infected host. A similar result (introduction of the marker gene without infection) may occur when a

35   transducing particle infects a target cell outside of the normal host range of the phage from which it was derived.

Transduction of the marker phenotype may take place via transient expression (i.e., expression from a gene which is not stably inherited by the cell) of the marker gene. In such case, the DNA transduced by the bacteriophage may not survive intact through the entire test period. However, transcription of the marker gene transduced by the phage will be sufficiently efficient before the DNA is degraded to ensure that the bacteria has assembled a functional marker by the end of the test period. The bacteria can thus be detected by the assay of the invention even if the bacteria degrades the phage DNA.

Bacteriophage useful in the present invention may be obtained from microbiological repositories, such as the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852. Virulent bacteriophage are available as bacteria-free lysates, while lysogenic bacteriophage are generally available as infected host cells.

Wild-type bacteriophage obtained from any source may be modified by conventional recombinant DNA techniques in order to introduce a desired primary marker gene capable of producing the detectable phenotype of interest. Prior to introduction, the marker gene of interest will be combined with a promoter region on a suitable gene cassette. The gene cassette may be constructed by conventional recombinant DNA techniques in a suitable host, such as E. coli. Both the marker gene and the promoter region should be chosen to function in the target host, and the cassette may optionally include a second marker gene, such as antibiotic resistance, heavy metal resistance, or the like, to facilitate in vitro manipulation.

The primary marker gene (or genes, if not a single gene system) should be capable of expressing an easily detectable phenotype in the target bacterial host. Suitable phenotypes include bioluminescence, enzyme-

catalyzed color production (e.g., using the enzyme β glucuronidase (GUS)), ice nucleation activity, and the like. Preferred is the use of ice nucleation activity which is demonstrated herein to be easily and rapidly detectable using conventional ice nucleation assays.

5   While the remainder of this disclosure is directed primarily at the introduction and detection of ice nucleation activity and target bacterial cells, the principles disclosed can be applied broadly to other

10 detectable phenotypes.

        Suitable ice nucleation genes may be isolated from microorganisms which naturally display an ice nucleation phenotype. Exemplary ice nucleation genes and the bacteria from which they may be isolated include inaZ

15 isolated from Pseudomonas syringae S203, inaY isolated from Pseudomonas syringae PS31, inaW isolated from P. fluorescens MS1650, and iceE isolated from Erwinia herbicola. The sequence of inaZ is given in Green and Warren (1985) Nature 317:645-648, while the sequence of

20 inaW is given in Warren et al. (1986) Nucl. Acid. Res. 14:8047-8060, the disclosures of which are incorporated herein by reference. See also U.S. Patent No. 4,464,473, the disclosure of which is incorporated herein by reference. The teachings of these references are

25 sufficient to enable one skilled in the art to isolated an ice nucleating organism from the wild and obtain an ina gene therefrom by conventional cloning and screening techniques.

        The transducing particles of the present

30 invention may be prepared by a number of conventional genetic manipulation techniques, including site-directed insertion of the marker gene cassette into the bacteriophage genome, packaging of the plasmid carrying the marker gene or a portion thereof into the

35 bacteri phage coat, transp son mutagenesis, and h m logous rec mbination. The choice among these alternatives depends on the nature of the bacterial host,

the nature of the bacteriophage, and the extent to which the bacteri phage has been characterized.

For well characterized bacteriophage, particularly those which have been genetically mapped, it is frequently desirable to place the primary marker gene cassette, including the promoter region and optionally the second marker, into the bacteriophage genome by standard recombinant DNA techniques. After preparing the plasmid in a suitable host as described above, the marker gene cassette is excised and inserted into the desired bacteriophage. Strategies for insertion into λ phage, which may be generalized to other bacteriophage, are described in Young and Davis (1983) Proc. Natl. Acad. Sci. USA 80:1194-1198, the disclosure of which is incorporated herein by reference.

Transducing particles capable of lytic or lysogenic infection are prepared by deletion of non-essential regions of the bacteriophage genome and substitution of the gene cassette. Desirably, the regions deleted and inserted will be approximately the same size so that the packaging may be effected with minimum disruption. In some cases, however, it may be necessary to delete certain essential regions of the bacteriophage genome, particularly when it is desired to insert relatively large marker gene cassettes. In that case, the transducing particles will retain the ability to insert the DNA into the desired bacterial host, but will be unable to reproduce within the host. Reproduction may be obtained, however, by providing a helper bacteriophage, such as the wild-type bacteriophage, which is able to provide essential packaging functions.

Alternatively, for well characterized bacteriophage, it is possible to package the plasmid r the marker gene cassette by attachment of the bacteriophag pac site in a DNA c nstruct with the plasmid or cassette. The pac site may be obtained fr m

the bacteriophage genome and cloned into the plasmid
carrying the primary marker gene, promot r region, and
optional s cond marker. The plasmid may then be
transferred to a suitable bacterial host which is then
infected with a bacteriophage having a defective pac
site. The bacterial host will then produce transducing
particles having the plasmid and/or marker gene cassette
packaged within a bacteriophage coat capable of inserting
the plasmid DNA into bacteria of its host range. General
methods for cloning pac sites and producing packaging-
deficient bacteriophage are described by Vogel and
Schmieger (1986) Mol. Gen. Genet. 205:563-567, the
disclosure of which is incorporated herein by reference.

For less characterized bacteriophage,
transposon mutagenesis may be employed to prepare the
transducing particles of the present invention. The
method of Spanova and Karlovsky (1986) Folia Microbiol.
31:353-363 for the mutagenesis of phage L (Salmonella
typhimurium), the disclosure of which is incorporated
herein by reference, may be generalized to apply to other
transposons, bacteriophage, and bacterial hosts. A
marker gene cassette from the plasmid, as described
above, is first inserted into a desired transposon by
conventional techniques. The modified transposon is then
transposed into the desired bacteriophage by simultaneous
infection of a suitable host with both the modified
transposon and the bacteriophage. The host cells are
then incubated until lysis occurs, and the released phage
are collected. A fraction of the released phage will be
carrying the transposon insert. Host cells free from
bacteriophage are then infected with the phage mixture
obtained as described above, and cells carrying a
detectable marker present on the transposon selected.
Unlysed selected cells, in which the bacteriophage
carrying the modified transposon has become lysogenic,
are then plated n selective media and screened for the
desired phen type. Such selected cells thus c ntain

prophage having the desired primary marker gene under the control of a suitable promoter. Transducing particles may then be obtained by inducing the prophage to a lytic state, which results in cell lysis and release of a large quantity of suitable transducing particles.

The use of a secondary selection marker in the preparation of transducing particles by transposon mutagenesis is helpful, but not required. Phage which have been mutagenized to carry the primary selection marker, for example the ice nucleation gene, may be identified over the background of wild-type phage by performing an appropriate assay, such as a replica plating assay for ice nuclei in the case of the ice nucleation gene. Such screening assays are taught in Lindow et al. (1978) App. Environ. Microbiol. 36:831-838, the disclosure of which is incorporated herein by reference.

In addition to the above techniques, transducing particles may be prepared by homologous recombination resulting in low frequency transducing particles or high frequency transducing particles. Low frequency transducing particles may be constructed from the plasmid carrying the marker gene cassette by first preparing a restriction library of the bacteriophage genome of interest. The restriction fragments from the library are then cloned into the plasmid in a suitable host, resulting in plasmids carrying relatively large regions homologous with the wild-type bacteriophage. Such regions serve as cross-over points in homologous recombination events in a manner well described in the art. The recombinant plasmid is then introduced to a suitable bacterial host, and colonies which express the primary or secondary marker gene are selected for infection with the wild-type bacteriophage. A relatively low percentage of the transducing particles produced by the bacteri phage infecti n will includ the entire plasmid carrying the marker gene cassette as an insert.

A fresh bacterial culture is then infected with the mixed transducing particles, and transformed colonies are selected based on the expression of either the primary marker or secondary marker carried by the plasmid.

5   A specific example of the preparation of low frequency transducing particles capable of specifically infecting S. typhimurium is given in the Experimental section hereinafter as Example 2.

Low frequency transducing particles having a
10   relatively high transduction frequency, usually greater than about 5%, may be used for performing the assay of the present invention. For transducing particles having a lower rate of transduction, it will usually be desirable to further modify the bacterial hosts to
15   produce high frequency transducing particles.

High frequency transducing particles are prepared as described above, but employing a prophage in the bacterial genome as the target for homologous recombination. To this end, a lysogenic strain (a strain
20   carrying the wild-type bacteriophage DNA in its genome as a prophage) is employed and transformed with a plasmid carrying the gene marker cassette which is incapable of replication in the host. Thus, only hosts where the selective markers have been incorporated in the prophage
25   will be viable under the appropriate selective conditions. Alternatively, the plasmid may be otherwise altered so that recombination with the prophage is readily detected. Plasmids incapable of replicating in the desired bacterial host may be prepared by several
30   techniques. For example, low frequency transducing phage prepared as described above may be utilized as the immunity functions expressed by the prophage will prevent replication. Second, plasmids capable of replication in a suitable cloning host but incapable of replication in
35   the target host f interest may be used. Finally, the plasmid may be modified so that the primary marker g ne is inserted into the prophage, while the secondary marker

is placed outside the region which is inc rporated in the prophag . R combination may then be det cted by screening for cells which express the primary selection phenotype, but which have lost the secondary selection phenotype. Incorporation of the desired marker gene into the prophage may be confirmed by standard techniques, such as Southern blotting as described in Southern (1975) J. Molec. Biol. 98:503-517.

A specific example of a high frequency transducing particle capable of specifically infecting S. typhimurium is given in the Experimental section hereinafter as Example 3.

A preferred means of making a high frequency transducing particle via homologous recombination involves the use of transposon tagging where an insertable element comprises the gene for a detectable phenotype of interest joined to the essential terminal sequences of a transposon. Such insertable elements will be able to identify sites in a bacteriophage genome into which an ice nucleation or other reporter gene can be inserted without interfering with essential bacteriophage functions. In general, any bacterial transposon can be used to prepare the insertable element. It is desirable that the size of the resulting insertable element be as small as possible to allow packaging of the entire resulting phase genome into mature viral particles.

As the frequency of occurrence of suitable sites in transposon tagging is expected to be low, the bacterial transposon used preferably encodes functions that allow for the selection of transposition events. To this end, a naturally occurring transposon may be modified to provide a small-sized, selectable transposon. Any transposon in which the transposase functions can be separated from the ends of the transposabl element w uld be suitable. These include but are not limited to IS (insertion sequence) elements, such as IS50, and transposable drug resistance elements, such as Tn3, Tn5,

and Tn7, as is known in the art. The bacterial
transposon Tn5 is most convenient for such modifications
as it has been characterized in great detail in the art.
Krebs and Reznikoff, (1986) J. Mol. Biol. 192:721-791;
5   Dodson and Berg, (1989) Gene 76:207-213.

As one approach, the essential terminal DNA
sequences of a transposon can be synthesized chemically
by methods known in the art. In synthesizing these DNA
sequences, it is advantageous to include flanking
10   sequences (polylinkers) that contain the recognition
sites for a number of restriction endonucleases. This
facilitates the insertion of selectable markers between
the ends of the transposon element, and allows the
element thus created to be easily cloned into a vector
15   plasmid, the choice of which depends on the nature of
bacterial host for the particular bacteriophage. As is
well known in the art, narrow host range plasmids derived
from ColE1, P15A, or pMB9 replicons (such as pAT273,
pACYC184, pBR322, or pUC19) would be suitable for use in
20   bacteria from the family Enterbacteriaceae, while in
general, any broad host range plasmid derived from the
incompatability groups IncP, IncW or IncQ (such as those
derived from pRK2, pS-a or pRSF1010) would be suitable
for use in other gram negative bacteria. For gram
25   positive bacteria other suitable plasmid vectors known in
the art can be used.

It is preferred that the transposase for the
chosen transposon not be located between the ends of the
transposon. In this way, once the transposable element
30   has been inserted in the bacteriophage genome, it will be
unable to undergo a second transposition event. In
general, it is also desirable that this location be on
the same DNA molecule as the element to be transported
(i.e., in cis), although the transposon and the
35   transposase could, in some systems, be located on
separate plasmid replicons (i.e., in trans).

It may also be desirable to modify the expression of the transposase gene of the transposable element by inserting the coding sequence for the transposase protein downstream of a highly expressing bacterial promoter. Such promoters are known to those of skill in the art, and include the tac, trc and lac promoters of E. coli. It is known that overexpression of bacterial transposases can be deleterious to the host cell, so it is also desirable that the transcription from the chosen promoter be capable of regulation. Promoters such as the tac, trc and lac promoters are repressed by the lac repressor, the product of the lacI gene. Inclusion of such a gene on the same molecule as the transposase will ensure expression of the transposase protein only under conditions in which repression is eliminated (i.e., inducing conditions). For the lacI gene, the chemical isopropylthiogalactoside (IPTG) tends to be lac repressor limiting.

In general, any combination of a highly expressing yet repressible promoter and its associated repressor gene will function to give the same result. If a selectable marker is used as an insert between the ends of the transposable element, the marker should be as small as possible. It is preferred that the size of the transposable element be less than 1 kilobase. In general, antibiotic resistance genes are larger than is desirable, although the absolute size of the transposed DNA is determined by the packaging limits of the individual bacteriophage.

A preferred means of selection uses the phenomenon of α-complementation to lactose utilization as described in E. coli. Here, a small fragment of the β-galactosidase gene (the lacZ α fragment which is approximately 500 base pairs in length) enc des a polypeptide which rest res functionality to an ther mutant polypeptide in which amino acids have been deleted (the M15 deleti n). The inclusion of the lacZ α fragment

between the ends of the transposon allows for the
selection of bacteriophage carrying the inserted DNA by
their ability to restore lactose utilization to host
bacteria which cary the lacZM15 deletion either on a

5   plasmid or in the chromosome. The DNA fragments encoding
these two activities have been characterized in detail
and can be readily manipulated by those skilled in the
art. See, I. Zabin et al., The Operon (eds. J.H. Miller
et al.), Cold Spring Harbor Laboratory (New York), 104-

10   107 (1978).

          A plasmid containing the transposase, end piece
DNA, marker DNA and repressor, as described above, is
introduced into a bacterial cell (e.g., Salmonella)
containing within the bacterial genome prophage DNA

15   (e.g., P22). The transposase is induced by an
appropriate chemical inducer (e.g., IPTG) and the
bacterial cells are grown for a number of generations.
The prophage is then induced, e.g., with an appropriate
chemical inducer (e.g., mitomycin C) yielding

20   bacteriophage. The bacteriophage which carry a
transposon insertion are selected by infecting a suitable
bacterial host (e.g., carrying M15 deletion) with the
induced phage lysate. Infected cells are plated out on
media selective for the marker (e.g., lacZ α), resulting

25   in selection of cells containing a prophage genome which
in turn contains the transposon fragment containing end
pieces and marker. The selected cells are then induced
for phage lysis to obtain pure phage preparations. The
DNA fragment of bacteriophage carrying the transposon

30   insertion is isolated using in vitro techniques. This
fragment is cloned on a suitable plasmid vector. The ice
gene is then substituted for the selectable marker (e.g.,
lacZ α) using in vitro techniques. Homologous
recombination can then be carried out using a plasmid

35   containing the ice gene so prepared, where the homologous
recombination inv lves recombinati n between prophage DNA
in the bact rial genome and prophage DNA flanking the ice

gene on the plasmid. The resultant strain, upon phage
induction, yields transducing particles containing ice
gene DNA at a site within the prophage DNA where the
presence of the ice gene at that site does not disrupt
essential phage functions.

Transducing particles prepared as described
above are used to detect target bacteria in biological
samples as follows. In some instances it will be
possible to infect a biological sample and observe the
alteration and phenotype directly, although in other
cases it may be preferred to first prepare a mass culture
of the bacteria present in the sample. Methods for
obtaining samples and (if necessary) preparing mass
culture will vary depending on the nature of the
biological sample, and suitable techniques for preparing
various sample types are described in detail in standard
microbiology and bacteriology texts such as Bergey's
Manual of Determinative Bacteriology (8th ed.), Buchanan
and Gibbons (eds.) Williams & Wilkens Co., Baltimore
(1974); Manual of Methods for General Bacteriology,
Gerhardt et al. (eds.), Am. Soc. Microbiology, Washington
(1981); and Manual of Clinical Microbiology (2nd ed.),
Lennette et al. (eds.), Am. Soc. Microbioloty, Washinton
(1974).

Once the biological sample has been prepared
(with or without growth of a mass culture), it will
typically be exposed to transducing particles under
conditions which promote binding of the particles to the
bacteria and injection of the genetic material, typically
at a temperature which supports rapid growth of the
bacteria (e.g., 35°C to 40°C) without agitation for a
time sufficient to allow infection (e.g., 15 minutes to
120 minutes). For transducing particles carrying an ina
gene, the sample is then incubated at a 1 wer temperature
(e.g., 20°C to 25°C) to permit f rmation of ice
nucelation sites. After a short time, typically 30

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minutes to 2 hours, the sample may be assayed for ice
nuclei as described in more detail below.

For transducing particles carrying an ice
nucleation gene, conventional droplet freezing assays as
5   described in Vali (1971), supra., may be useful to detect
transformation to the ice nucleation phenotype.
Alternatively, a specific fluorescence freezing assay for
detecting the ice nucleation phenotype is taught in U.S.
Patent No. 4,784,943, the disclosure of which is
10   incorporated herein by reference. Briefly, the assay for
detecting ice nucleation comprises use of fluorescent
compounds which in aqueous state manifest a change in
fluorescence or visible properties upon freezing or
thawing of the aqueous medium. Suitable fluorescent
15   compounds may be selected from the fluorescein family,
e.g., calcein, carboxyfluorescein, and related compounds.

As described above, the assay of the present
invention is useful for screening biological samples to
20   determine whether bacteria present in the host range of
the transducing particle are present. The present
invention is also useful for typing bacterial species and
strains in a manner similar to conventional phage typing
which relies on plaque assays for determining phage
25   infection.

For typing according to the present invention,
a panel of transforming particles having differing,
usually overlapping, host ranges are employed. The
species and strain of the target bacteria may then be
30   determined based on the pattern of reactivity with the
various transforming particles. Often, such tests may be
run on a single carrier, where the different transforming
particles are spotted in a fixed geometry or matrix on
the carrier surface. The pattern of reactivity may then
35   be rapidly observed.

The present invention may be combined with
nutritional scre ning in order to further characteriz

the bacteria being investigated. By providing a selective medium during either the mass culture or the plating culture, the range of bacteria which can remain viable may be limited. As the phenotypic assay of the present invention can only detect viable cells, absence of a detectable phenotype limits the type of bacteria which may be present. By properly combining the host range of the transducing particles and the viability range of the selective medium, the method of the present invention can be made very specific for the type of bacteria being determined.

A second approach for increasing the ability of the present invention to specifically identify bacterial hosts involves the use of immunoadsorption. Immobilized antibodies, including antisera or monoclonal antibodies, are utilized to specifically capture bacterial cells based on the identity of their cell surface epitopes. The bacteria may then be further detected using the transducing particles of the present invention, as described above. Suitable materials and methods for the immunoadsorption of particular bacterial species and strains on solid phase surfaces are described in Enterobacterial surface antigens: Methods for molecular characterization, Korhonen et al. (eds.), Elsevier Science Publishers, Amsterdam (1985).

The present invention can be particularly useful in patient diagnostics as it allows the determination of bacterial sensitivity to antibiotics and other bactericides. By performing a short incubation of the bacteria with an antibiotic or bactericide to be screened prior to exposure to the transducing particles of the present invention, the metabolic activities of the cells will be halted and the alteration of phenotype prevented. Such testing will be useful after the presence of the bacteria is initially c nfirmed using the transforming particles as described ab ve. Antibiotics and bactericides which are determined to be lethal to the

bacterial infection may then be employed for treatment of
the patient. Such rapid and early detection of useful
antibiotics and bactericides can be invaluable in
treating severe bacterial infections.

In a specific embodiment, a means is provided
for assaying bacteria which have been previously rendered
susceptible to bacteriophage of the invention on a phage-
specific basis. That is, in a first step, the target
bacteria are modified, e.g., by transformation, so that
they contain or express a cell-specific receptor for the
bacteriophage of interest. In a second step, the
modified (or tagged) bacteria are introduced into, or
mixed into, a sample environment in which they are to be
followed. The sample environment can be any setting
where bacteria exist, including outdoors (e.g., soil, air
or water); on living hosts (e.g., plants, animals,
insects); on equipment (e.g., manufacturing, processing
or packaging equipment); and in clinical samples. The
bacteriophage assay of the invention (as described
previously) can then be carried out, using bacteriophage
specific for the introduced receptor, and the presence of
the tagged bacteria can be monitored or quantified.

An advantage of this embodiment is that it
provides a means to follow or track bacteria to be
released into a sample environment which already contains
the same type of bacteria (or closely similar bacteria)
or which may be subject to introduction of the same type
of bacteria (or closely similar bacteria) from a separate
source. The bacteria being tracked can be distinguished
from the other bacteria (i.e., bacteria which are
essentially the same) by virtue of the presence of the
cell-specific receptor which has been introduced into the
bacteria being tracked. There is thus provided the
opportunity of assaying for the presence of released
bacteria in the presence of otherwise identical (but for
the receptor comp nent) bacteria, without cross
reactivity (backgr und).

An exemplary approach for monitoring
Pseudomonas uses the lamB gene of E. coli which is known
to be a receptor for the coliphage λ. See, G. Vries et
al. (1984) Proc. Natl. Acad. Sci. USA 81:6080-6084, and
R. Ludwig, (1987) ibid. 84:3334-3338, both of which are
incorporated herein by reference. Expression of lamB
renders Pseudomonas species susceptible to attachment of
λ phage and injection of phage DNA. A recombinant λ
phage carrying a reporter gene, e.g., an ice nucleation
gene, usually under the control of a strong promoter, is
constructed in a broad host range plasmid. A lamB gene
is inserted (e.g., by homologous recombination) into the
chromosome of the bacteria to be assayed. The assay is
then conducted in accordance with the teachings herein.

One specific use of this approach is to monitor
Pseudomonas bacteria (e.g., P. syringae), including
Pseudomonas soil bacteria and Pseudomonas epiphytic
bacteria, which are released into a specific environment
or setting, e.g., soil, a greenhouse or field setting.
At least some of the bacteria to be released are first
transformed to express the bacteriophage-specific
receptor. Then by collecting bacteria from the
environment at a later time, the presence of the bacteria
in the environment can be determined. The approach
provides a means to follow the presence, the migration
and the survivability of the bacteria.

The following examples are offered by way of
illustration and not by way of limitation.

EXPERIMENTAL

1. Assay for Male (F⁺) E. coli.

INA transducing particles were prepared as
follows. The pUC118 plasmid-M13 bacteriophage system
(Vieira and Messing (1987) Meths. Enz. 153:3-11) was
modified by inserting ice nucelati n gene inaY (isolated
from Pseudomonas syringae strain PS31, which strain is
described in Deininger et al. (1988) J. Bacteriol.
170:669-675), into plasmid pLVC76; this plasmid also

carries an M13 bacteriophage pac site. Transducing
particles were then produced using a packaging-defective
M13 helper phage. The inaY gene was under the control of
a lac promoter derived from the original pUC118 plasmid,
and the resulting transducing particles retained the
specificity of M13 phage for male (F') strains of E. coli.

The transducing particles were passed through a
0.2 μm filter (to remove any INA⁺ cells remaining in the
preparation) and found to be free from ice nucleation
activity down to -12°C. Efficiency of transformation of
E. coli MV1193 (F') was measured independent of ice
nucleation activity based on the activity of an
ampicillin resistance gene carried by pLVC76. Plating
assays of cells (100 μl; OD_{600}=0.001) exposed to the INA
transducing particles (10 μl) were performed by
incubation at 37°C for 30 min., dilution to 1 ml, and
plating of 50 μl aliquots. The results are set forth in
Table 2.

TABLE 2

Transfecting Particle Dilution	Colonies Recovered (- ampicillin)	Colonies Recovered (+ ampicillin)
10^0	404	45
10^{-1}	792	45
10^{-2}	865	6
10^{-3}	838	1
10^{-4}	equivalent to 10^{-3}	0

The transducing particles produced two effects.
First, they reduced cell viability. Such reduced
viability was probably caused by helper phage, the
packaging-defective phage which were used to construct
the particles, and which contaminate the transducing
particles. Second, they transformed some cells to
ampicillin resistance. Five of the ampicillin-resistant
colonies were checked for ice nuclei; all colonies tested
were INA⁺. If the number of colonies recovered at low
phage concentrations, in th absence of ampicillin, is

used to estimate the number of bacteria originally present, then the transformation efficiency at high phage concentrations was about 5%.

Samples of *E. coli* MV1193 were tested as follows. Cells (100 μl, OD_{600}=0.01) were incubated with 10μl of a suspension of INA transducing particles (10· dilution) at 37·C for 2 hours, then at room temperature for 1 hour. The samples were then tested for ice nucleation activity in a standard droplet freezing assay consisting of 40 x 10 μl drops per dilution, with dilution by decades from 10^{-1} to 10^{-5} of the original sample concentration. The results of the freezing assay are set forth in Table 3.

TABLE 3

T(·C)	Nuclei per ml (no TP's)[1]	Nuclei per ml (no cells)	Nuclei per ml (cells + TP's)	% Nuclei per original cells[2]
- 3.7	0.0	0.0	3.0×10^1	0.002 %
- 4.1	0.0	0.0	6.9×10^2	0.099 %
- 4.6	0.0	0.0	1.6×10^3	0.23 %
- 5.2	0.0	0.0	8.2×10^3	1.2 %
- 6.2	0.0	0.0	1.6×10^4	2.3 %
- 7.3	0.0	0.0	2.4×10^4	3.5 %
- 8.2	0.0	0.0	2.5×10^4	3.6 %
- 9.3	0.0	0.0	1.4×10^5	19.8 %
-11.1	0.0	0.0	3.0×10^5	42.8 %

[1]   TP's are transducing particles.

[2]   The original concentration of cells was calculated using the approximate relationship that an OD_{600} of 1.0 is 7.0×10^8 cell/ml. The detection limit was 5 nuclei/ml.

After 3 hours, the test measured the number of cells in a dilute suspension, with a sensitivity (approx. 40%) approaching that of a plating assay (approx. 90%). The plating assay requires about 15 hours f r *E. coli*. The background was below the limit f detection. Therefore, the signal:noise ratio was at least 10^5. The data sh wed the temperature-dependent sensitivity which

is typical of ice nucleation assays, with the data point
at -11°C being indicative of the total number of cells
transformed to the INA⁺ phenotype.

In order to test for biological specificity of
the INA transducing phage, an F⁻ strain of *E. coli*
(JE5505, a K12 derivative) was tested using the same
protocol described above for E. coli MV1193. Attempted
transduction of the *E. coli* JE5505 gave rise to no
detectable nuclei. Antibiotic sensitivity or resistance
was tested for as follows. The experiment was
essentially the same as that described in connection with
Table 3, except that the bacteria were first incubated
with tetracycline (10 μg/ml), chloramphenicol (50 μg/ml)
or no antibiotic (control) for 30 min at 37°C. The
results of this experiment are set forth in Table 4.

TABLE 4[1]

T(°C)	Nuclei per ml (+ tetracycline)	Nuclei per ml (+ chloramphenicol)	Nuclei per ml (control)
- 3.6	1.1×10^1	0.0	0.0
- 4.0	2.9×10^2	0.0	1.1×10^2
- 4.7	4.3×10^3	0.0	4.3×10^3
- 5.4	9.2×10^3	0.0	1.1×10^4
- 6.3	1.8×10^4	0.0	2.0×10^4
- 7.2	3.2×10^4	0.0	3.0×10^4
- 8.3	4.3×10^4	0.0	3.2×10^4
-10.2	3.0×10^5	0.0	3.0×10^5

1   Test conditions were as for Table 3, except that cells were
preincubated for 30 min. at 37°C with the antibiotic shown,
before the addition of the INA transducing particles.

The assay correctly measured the number of
tetracycline resistant bacteria in the sample, and
correctly determined that all of these bacteria were
chloramphenicol-sensitive. Thus, the assay of the
present invention is suitable to screen for antibiotic
r sistance and sensitivity.

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2.   <u>Assay for *Salmonella typhimurium*.</u>

A low frequency transducing particle capable of
conferring ice nucleation activity was obtained by
homologous recombination of plasmid pRLG61 with
bacteriophage P22. Plasmid pRLG61 contains the ice
nucleation gene *inaW* (isolated from *Pseudomonas
fluorescens* MS1650 as described in Corotto et al. (1986)
EMBO J. 5:231-236) under the transcriptional control of
the tac promoter (Bagdasarian et al. (1983) Gene 26:273-
282). The origin of replication and an ampicillin
resistance gene are derived from pBR322 (Bolivar et al.
(1977) Gene 2:95-113), and pRLG61 contains a 4.3 kb
insert of P22 DNA which was cloned from a *Hind*III digest
of the P22 chromosome. pRLG61 was constructed *in vitro*
and cloned in *E. coli*. Using a plasmid mobilization
system, it was then transferred by conjugation into
Salmonella typhimurium strain LT2. An ice nucleation
assay for the pRLG61 (LT2) strain (designated RGS1)
demonstrated a high level of ice nucleation activity.

INA transducing particles were prepared by
infecting a liquid culture of RGS1 with wild-type P22
phage at a multiplicity of infection (m.o.i.) of 5 plaque
forming units (PFU) per cell. The infected culture was
incubated several hours and a raw phage-containing lysate
prepared by treatment of the culture with chloroform (to
lyse bacterial cells) followed by centrifugation (to
pellet cell debris). The phages remained in the
supernatant fraction along with various cellular
material, including still-active ice nuclei. Subsequent
centrifugation through a cesium chloride block gradient
separated phage particles (which were collected from the
3M/5M interface) from the ice nuclei and other
proteinaceous cellular contaminants (Davis et al. (1980)
Advanced Bacterial Genetics, Cold Spring Harbor
Laboratory, Cold Spring Harbor, NY, pp 80-82). Finally,
the phage-c ntaining fracti n was dialyzed to remove the

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cesium chloride, then filtered through a 0.45 μm filter
to sterilize the preparation.

Due to the nature of their formation, the
proportion of transducing particles to wild-type phages
5   in such a preparation was small. The detection of ice
nucleation activity and the recovery of drug-resistant
transductants from a transfected culture (see below) both
indicated that the transducing particle preparation was
able to generate a single transduction event for
10 approximately each 1000 normal lytic infections.

A preparation of low-frequency transducing
particles (derived by infection of RGS1 with P22 as
described above) was used to transduce the *inaW* gene into
a culture of *S. typhimurium* LT2. Relatively high-density
15 cultures were used to help protect transduced cells from
lethal super-infection by the wild-type phages which are
the majority species in the transducing particle
preparation. Additionally, ampicillin (Ap) was added to
the medium following phage adsorption to the host cells,
20 to further reduce the rate of lytic infections with wild-
type P22 (neither the host cells nor the wild-type phages
were resistant to Ap). The species-specificity of the
transducing phages was tested by parallel transfection of
E. coli strain AB1157 with the transducing particle
25 preparation.

Late log-phase cultures (OD_{600}=0.5 - 1.0) of
S. typhimurium LT2 and *E. coli* AB1157 were infected with
the transducing particle preparation at an m.o.i. of
approximately 0.1 PFU per cell. Cultures were incubated
30 at 37°C without agitation and particles were allowed to
adsorb to host cells for a period of 20 min. Control
samples were run in parallel and consisted of sterile
broth "infected" with transducing phages and uninfected
bacterial cultures. Ap was added to the infected
35 cultures, and incubation continued for an additi nal 40
min. at 37°C with agitation. Cultures were then

incubated at 24°C for 1 hour to allow expression of ice
nucleation activity.

Ice nucleation assays were then performed as
described above for *E. coli* in connection with Table 3.
OD_{600} of the LT2 and AB1157 cultures was measured at 1.0
and 0.67, respectively. The results are set forth in
Table 5 below.

<p align="center">TABLE 5</p>

T(°C)	TP's no cells	LT2 uninfected	AB1157 cells+TP's	LT2 cells+TP's	% INA⁺ LT2 cells+TP's
- 5.7	0	0	0	8.7×10^1	1.2×10^{-5}
- 6.0	0	0	0	2.9×10^2	4.1×10^{-5}
- 7.0	0	0	0	1.8×10^3	2.6×10^{-4}
- 8.0	0	0	0	8.7×10^3	1.2×10^{-3}
- 9.0	0	0	0	3.9×10^4	5.6×10^{-3}
-10.0	0	0	0	2.1×105	3.0×10^{-2}
-11.0	0	0	0	2.7×10^5	3.9×10^{-2}
-12.0	0	0	0	2.7×10^5	3.9×10^{-2}

The table presents the ice nucleation activity
of experimental samples as a function of temperature.
The cumulative frequency of ice nuclei per ml of original
culture is give for particular temperature points. The
last column (% INA⁺ LT2) represents the number of ice
nuclei per ml expressed as a percent fraction of the cell
population, estimated to be 7×10^8 cells/ml for OD_{600}=1.0.
The above results demonstrate that the
expression of ice nucleation activity occurs only as a
result of the infection of a susceptible host strain
(LT2) with the transducing phage preparation. Neither
the host cells nor the phages by themselves express any
detectable ice nucleation activity. Furthermore, this
experiment has confirmed the host strain specificity of
the particles is maintained after transducti n of the
inaW gene, since no detectable transduction of ice
nucleation activity was observed in *E. coli* AB1157. As

an indication of the transduction frequency, the maximum frequency of ice nuclei observed in the above experiment, 2.7×10^5 per ml, corresponded to 1×10^{-3} ice nuclei per PFU of the transducing phage preparation.

Transduction of plasmid pRLG61 was also detected by recovery of ampicillin-resistant colonies from the transfected culture. After infection and initial incubation as described above, the cells were spread on agar medium containing ampicillin. The agar surface was previously coated with a layer of heat-killed LT2 to adsorb and thus protect recipient cells from the lethal wild-type phages in the preparation. Ampicillin-resistant transductant colonies appeared after overnight incubation. The results indicated a transduction frequency for pRLG61 of 6×10^{-3} Ap^r colonies per PFU of the transducing phage preparation. Thus, two separate experiments have determined the frequency of transduction of this system to be in the range of 10^{-3} to 10^{-2} transduction events per PFU of the transducing particle preparation.

In summary, low-frequency transducing particles were prepared by transduction of ice nucleation gene into phage P22. Particles so produced were then transduced into *Salmonella typhimurium* strain LT2 and found to confer readily detectable ice nucleation activity. Background ice nucleation activity in experimental control samples was found to be absent, and no ice nucleation activity resulted upon the attempted transfection of a bacterium, *E. coli*, which is non-permissive for P22.

3. Phage P22 High Frequency Transduction System

The following describes a high frequency transduction (HFT) system which was developed using phage P22. This system allowed the high-frequency transduction of the *inaW* gene t susceptible strains of *Salmonella*.

The HFT system was constructed so that the transduced reporter gene (the *inaW* ice nucleation gene

from *Pseudomonas fluorescens*) existed as a permanent cointegrate with a P22 prophage, contained in a lysogenic strain of *Salmonella* which was used to produce the high-frequency transducing particles/phages (HFTPs).

Propagation of the HFTP-producing strain resulted in a culture in which every cell was a potential producer (upon induction of the recombinant prophage's lytic cycle) of TPs, yielding a high proportion of TPs to non-transducing, wild-type revertant phages.

Two basic components were used to construct the HFT system: i) a *polA* mutant strain of *S. typhimurium* (defective for DNA polymerase I), and ii) plasmid pRLG68, a mobilizable plasmid encoding ice nucleation activity and ampicillin (Ap) resistance, with a ColE1-type origin of replication.

The *polA* strain, called AA3007 (Whitfield and Levine, (1973) J. Bacteriol. 116:54-58), was used specifically because it is unable to support the autonomous replication of ColE1-derived plasmids. To facilitate its use in subsequent manipulations, a spectinomycin-resistant (Sc^r) derivative of AA3007 was first selected as follows: A culture of AA3007 was grown in 5 ml L-broth (LB) overnight (ON) at 37°C; 2 ml of the ON culture was used in turn as inoculum for a 200 ml L-broth culture which contained Sc at a concentration of 100 ug/ml. After growth for 24 h., replicate platings of the culture were made on LB+Sc (Sc at 50 ug/ml) plates, using 50 ul of the 24 h. liquid culture per plate. Sc^r colonies were identified and isolated after 24 h. growth of the plates at 37°C. One such Sc^r colony was the origin of the AA3007 Sc^r strain, hereafter designated RGS10. Growth of P22 on RGS10 was confirmed, and a derivative which had become lysogenic for P22, called RGS11, was isolated by streaking cells from the turbid center of a P22 plaque formed on a lawn of RGS10.

Another key element of the HFT system was plasmid pRLG68, which was designed to facilitate the

incorporation (via homologous recombination) of a highly-
expressed *inaW* (ice nucleation) gene into the P22
prophage of strain RGS11. pRLG68 was constructed in two
steps. First, a clone of *inaW*, obtained on a 4.6 kb
BamHI/HindIII restriction enzyme fragment, was cloned
into the expression vector plasmid pKK223-3 (Brosius and
Holly (1984) Proc. Natl. Acad. Sci. USA 81:6929-6983).
pKK223-3 contains the ampicillin-resistance (Ap^r) gene,
origin of replication, and "bom" site (which allows it to
be mobilized by certain bacterial conjugation systems) of
pBR322 (Bolivar et al. (1977) Gene 2:95-113); in
addition, it contains the powerful *tac* promoter (de Boer
et al. (1983) Proc. Natl. Acad. Sci. USA 80:21-25)
situated so as to provide promotion to foreign genes
inserted proximally in the plasmid's polylinker (multi-
cloning-site) region. The intermediate *inaW*/pKK223-3
construct was called pRLG66. The second and final stage
of the construction of pRLG68 involved the insertion of a
fragment of P22 chromosomal DNA into a site in pRLG66.
The 2.5 kb P22 fragment known as *BamHI* fragment "B" in
the P22 physical map (Rutila and Jackson (1981) Virology
113:769-775) was purified from a *BamHI* digest of the P22
chromosome, and inserted in the unique *BamHI* site (just
upstream of the *tac* promoter region) of pRLG66 so as not
to interrupt any essential plasmid functuations, nor
interfere with the expression of *inaW*. The P22 fragment
was chosen for its lack of genes essential to the basic
life cycle of P22, since it would ultimately be the point
of insertion of foreign DNA in the P22 prophage during
the construction of the HFTP-producing strain. The
resulting P22/pRLG66 construct was called pRLG68.

       The *bom* region of pRLG68 (derived ultimately
from its pBR322 predecessor) allowed it to be mobilized
and transferred during bacterial conjugation, so long as
conjugation and mobilizati n functions were pr vided *in
trans* by the conjugal donor strain. Thus, pRLG68 was
transformed into the *E. coli* strain GJ23, in which it was

mobilizable by the R64drd11/pGJ28 system (Van Haute et al. (1983) EMBO J. 2:411-417), and from there transferred via inter-sp cies conjugation to RGS11. Since pRLG68 could not autonomously replicate in the recipient RGS11 strain (due to the *polA* mutation), any Ap^{r}Sc^r progeny from the mating were assumed to contain pRLG68 insertions in the P22 prophage, resulting from homologous recombination between the plasmid's region of P22 DNA and the homologous region in the prophage. A Southern blot analysis of a few representative clones (alongside their AA3007 and RGS11 progenitor strains) gave results consistent with the formation of such a pRLG68/P22 cointegrate. The resulting strain was called RGS12, and was the strain from which HFTPs were produced.

HFTPs were produced from RGS12 by treatment of cultures of the strain with Mitomycin-C, a drug which is known to induce the lytic cycle in lysogens of P22 and other such phages (Arber et al. (1983) in *Lambda II*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp 443-445). Such induction of RGS12 resulted in the replication and packaging of progeny phage particles, many of which were recombinant HFTPs. A culture of RGS12 was grown in L-broth at 37°C to mid-exponential phase (A_{600}=0.40), at which point Mitomycin-C was added to a final concentration of 5 mM. The culture flask was covered with foil to block any light that would stimulate the bacterial photo-active DNA repair mechanism (thus possibly compromising the efficiency of induction), and incubation resumed for several hours until the bacterial cells had lysed completely. Bacterial debris was cleared from the lysed culture by centrifugation at 2,000xg for 20 min., and the supernatant was sterilized by passage through a 0.45 um filter. Additionally, the RGS12 supernatant was heated 15 min. at 55°C, as this was found to destroy residual ice nucleation activity (as assayed by a standard droplet-freezing assay of the material) without adversely affecting the HFTPs themselves. As an

experimental control, phages were produced from RGS11
(the non-recombinant, lysogenic progenitor strain of
RGS12) in an identical manner.

Some experiments were done to characterize the
RGS12 HFTPs. First, the titers of phage preparations
from the mitomycin-C-induced RGS11 and RGS12 cultures
were compared with a sample of phages produced from a
similarly treated, non-mutant LT2(P22)$^{\cdot}$ lysogenic strain
(LT2 is a vigorous, non-mutant ancestor strain of RGS11
and RGS12). The titers were approximately 10^9, 10^8 and
10^7 PFU/ml for the LT2(P22)$^{\cdot}$, RGS11 and RGS12
preparations, respectively. Since phage titers are
measured by counting plaques formed on a bacterial lawn,
only phages able to undergo lytic infection are
assayable. The relatively low titer of the RGS12
preparation indicated a significant proportion of TPs,
since most TPs will be defective for lytic growth (the
heterologous DNA which they contain would crowd out
essential genes). This was confirmed by the
electrophoretic analysis of restriction digests of
purified RGS11 and RGS12 phage DNAs, which showed the
presence of extra restriction fragments in the RGS12
phage DNA. The sizes of the extra fragments corresponded
with those predicted for the pRLG68/P22 prophage
cointegrate; moreover, their relative intensities showed
that most of the phage particles contained the extra DNA.

The following work used the same preparation of
RGS12 HFTPs, prepared as described above. The detection
of bacterial cells by the transduction of ice nucleation
activity (INA) using RGS12 HFTPs will hereafter be
referred to as a "transduction assay". The transduction
assay procedure involved the infection, with HFTPs, of
samples which contain bacteria, and the eventual
determination of ice nucleation activity (INA) expressed
in the samples. Initial experiments were done to
characterize and optimize the detection of bacteria using
the HFTP system. Eventually, experiments were done to

simulate the assay of bacteria in real food samples, such as the example described below.

The following experiment was done to demonstrate the detection of *Salmonella* present in a whole egg mixture. A culture of *S. typhimurium* strain LT2 was grown in L-broth at 37°C to an optical density (A_{600}) of 0.35. The culture was then diluted in a series of 10-fold increments into raw, blended egg (i.e., the yolk and white were homogenized together); the dilution series in egg extended to 10^{-5} relative to the original ($A_{600}=0.35$) culture. Bacteria were assayed by transduction using RGS12 HFTPs as follows: 40 ul samples of each of the 10^{-2} to 10^{-5} bacterial dilutions in egg were added to 360 ul of sterile L-broth, which constituted a 10-fold dilution of the original samples. Additionally, control samples of the un-inoculated egg mixture were prepared in a similar manner (diluted 1:10 in LB). To each sample was added 100 ul of RGS12 HFTP preparation (which contained 2×10^6 pfu total), except for one of the un-inoculated egg controls, which instead received an equivalent amount of sterile L-broth. The samples were then incubated for 1 h. at 37°C followed by 1 h. at 23°C, to allow expression of transduced INA. The INA of transduced samples was then measured by a droplet freezing assay as follows. To each transduced sample was added 1/100 volume of a fluorescent dye mixture (100 mM carboxy-fluorescein, 100 mM tris-HCl pH 7.0) which aided in the detection of freezing events during the assay (the dye color changes from fluorescent yellow to dull red upon freezing). Dilutions of each sample were then made in decades from 10^{-1} to 10^{-5}) into the same carboxyfluorescein buffer (at 1 mM strength in all dilutions). $20 \times 10 \mu l$ droplets of each dilution ($10^\cdot$ to 10^{-5}) of the original assay samples (which were themselves transduced dilutions of the original bacterial culture) were then placed on paraffin-c ated foil which floated on the surface of a temperature-c ntrolled chilling bath.

The freezing of droplets was then monitored as the
temperature of the bath was lowered from -2.0°C to -
12.0°C over a period of 80 minutes. The raw frozen
droplet data was then used to calculate the frequency of

5   ice nuclei in the original samples (Vali (1971) J. Atmos.
Sci. 28:402-409). In its final form, the data are
expressed as a temperature spectrum of cumulative INA,
i.e., as numbers of ice nuclei per ml at a range of
temperature points. The results for the above experiment

10   are presented in Table 5.

TABLE 5[1]

Sample	Bacteria/ml	-6°C INA	-12°C INA
1. Un-inoculated Egg (no HFTPs)	0	0	0
2. Un-inoculated Egg + HFTPs	0	0	0
3. 10^{-2} Culture/Egg + HFTPs	2.5×10^5	3.0×10^4	6.9×10^5
4. 10^{-3} Culture/Egg + HFTPs	2.5×10^4	1.3×10^3	4.7×10^4
5. 10^{-4} Culture/Egg + HFTPs	2.5×10^3	1.6×10^2	4.5×10^3
6. 10^{-5} Culture/Egg + HFTPs	2.5×10^2	5.1×10^0	1.6×10^1

[1] The above data shows the results of INA assays performed on
transduced samples. Bacteria/ml was estimated from the A_{600} of
the original culture (according to the equivalency
$A_{600}=0.7=5 \times 10^8$ cells/ml), taking into account the 1:10 dilution
of the bacteria/egg mixtures into LB to prepare the samples for
transduction.

As shown in Table 5, the transduction of INA
using the HFT system allowed the detection of LT2 cells
in all samples in which they were present, even at as low
a concentration as 250 cells/ml (after dilution in LB
prior to the addition of HFTPs). Also apparent from the
un-inoculated egg samples is the absence of background
activity where no bacteria were present. The INA

observed in such experiments had a strong event-counting natur , in that individual INA˙ cells were detected. One simplication of this is that, since a majority of cells in a sample were transduced to the INA˙ phenotype, the frequency of INA of the sample allowed the estimation of the bacterial cell density. Thus, the measurement of INA at -12°C (at which temperature the detection of INA is the most sensitive) was strongly correlated with the population of LT2 cells in the sample.

In summary, a high frequency transduction system was developed in phage P22, in which the transduction of a *tac*-promoted *inaW* gene was maximized by manufacturing transducing particles from a *Salmonella* strain containing an ice nucleation gene in a P22 prophage. After verification of its structure by DNA analysis techniques, the HFT system was used in a transduction assay to detect LT2 cells present in samples of inoculated raw egg. The transduction assay showed very high sensitivity in detecting sparse populations of bacteria, with no background signal, and demonstrated that the assay could be used to estimate the number of bacterial cells present.

4.   Transduction in Heat-Disabled Cells

This example describes a set of experiments designed to test the response of disabled bacteria to phage transduction, and to determine how much recovery time may be necessary before such bacteria become detectable by the method.

Cell damage was induced in cultures of *Salmonella typhimurium* LT2 by exposure to injurious high temperature, during which time aliquots were removed at successive time points and plated on L agar (a rich, nonselective medium) and SS agar (a stringently selective medium used in the isolation of *Salmonella* and *Shigella*). After overnight incubation, the relative numbers of colonies which grew on L and SS agars for each heat-treated aliquot were used to gauge the extent of damage

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caused by the heat tr atment. The experiment below
describes the results of transduction of heat damaged
cells using the high frequency transduction (HFT) system
described in previous example (Example 3).

5   .   In the following experiment, a dilute culture
of LT2 was exposed to 55°C by immersion in a water bath.
Sample aliquots were removed from the culture at time 0
(i.e., just before immersion in the water bath), and at
times 60, 90, 120 and 150 seconds following immersion.

10   Dilutions of the aliquots were plated on L and SS agars
immediately, to allow the assessment of the initial
damage done to the bacteria. Each aliquot was then
divided to make two identical sets of heat-treated
aliquots. One set was transduced using RGS12 transducing

15   phages using a method (as described in Example 3) which
involved a total of 2 hours of incubation: 1 hour at 37°C
followed by 1 hour at 23°C. The other set of aliquots
was not transduced, but was incubated in parallel with
the transductions as a control for the recovery of the

20   heat disabled cells during the transduction procedure
(this observation would have been obscured in the phage-
infected aliquots). Dilutions of the untransduced
control aliquots were then plated on L and SS agars at
the end of the transduction incubations, while assays

25   were done to measure the INA expressed by the transduced
sample aliquots. The results of the experiment are
presented in Table 6.

TABLE 6

	Seconds at 55°C				
	0	60	90	120	150
Initial cfu/ml (LB)	1.4×10^5	1.1×10^5	9.1×10^4	3.5×10^4	7.0×10^3
Initial cfu/ml (SS)	1.4×10^5	9.7×10^4	8.0×10^3	< DL	< DL
Final cfu/ml (LB)	1.2×10^6	4.4×10^5	1.0×10^5	1.7×10^4	4.0×10^3
Final cfu/ml (SS)	1.4×10^6	4.3×10^5	7.0×10^4	5.0×10^3	< DL
Initial % Disabled	0	12	91	> 97	> 97
Final % Disabled	0	2	30	71	> 25
-12°C Ice Nuclei/ml	8.0×10^4	7.4×10^4	5.0×10^4	1.3×10^4	5.1×10^2

[1] Comparison of plate counts and INA of sample aliquots taken
from heat-disabling experiment. Colony counts on individual
plates have been translated into colony forming units (cfu) per
ml as for the undiluted culture, so as to be directly
comparable with INA data. "< DL" indicates where colony counts
fell below the detection limit (i.e., no colonies were detected
at the dilutions plated); DL for the platings of this
experiment was 1×10^3 cfu/ml. "% Disabled" values were figured
as 100-% (cfu on SS / cfu on L); when no colonies had been
observed on SS plates, the highest value below the detection
limit for the platings was substituted in the calculation.

One obvious effect of the heat treatment was
the outright killing of cells, which reduced the viable
cell population by 95% by the end of the time course.
Also apparent was the increasing proportion of disabled
cells among survivors collected at later time points;
i.e., progressively fewer of the LB-platable cells were
able to grow on SS agar. As can be seen in Table 6,
aliquots removed after 90 sec. contained mostly r
entirely disabled cells. Especially in these latter
samples, the effects of the heat treatment persisted to
the end of the transducti n procedure; the cultures

failed to divide and apparently, in the 120 sec. and 150
sec. aliquots, may have suffered additional mortality
during the following 2 hours. However, despite the
absence of growth in the severely damaged aliquots, there
was some degree of recovery from the disabled state
during the time taken to perform the transduction assays.
The degree of recovery was less in the more severely
damaged samples, in which a majority of cells initially
disabled remained in that state to the end of the
procedure.

The results presented in Table 6 indicate that
the frequency of ice nuclei expressed in the transduced
samples corresponded closely to the respective
populations of viable bacteria (i.e., cells which
survived heat treatment in either a disabled or non-
disabled state); this was true even when the initial
population consisted entirely of disabled cells. An
important corollary to this is that cells which had been
killed by the heat treatment were invisible to the assay.
In short, the response of heat-disabled bacteria to
detection by transduction was virtually the same as that
of healty cells. These results show that the phage
transduction assay may be performed without the need for
a recovery period to ensure the detection of disabled
cells.

To summarize, cell damage was induced in
cultures of _S. typhimurium_ strain LT2 by exposure to 55°C
for varying lengths of time. Platings of the heat-
damaged samples or LB agar and SS agar were then done;
the difference in the numbers of bacterial colonies which
grew on the two media was used as a measure of damage to
the bacterial culture. Transduction assays were then
performed on such heat-disabled bacteria using the RGS12
HFTP system. The results of the assays indicated that
disabled bacteria were detected with equal efficiency to
healthy bacteria, and that bacteria which had been killed

by the treatment became invisible to detection by the assay.

5.   The Host Range Specificity of a P22
     Transducing Phage

The following experiments were done to test the response of a P22-derived transduction system in assaying a number of different *Salmonella* serotypes from various serogroups. The "high-frequency transduction" (HFT) system described in Example 3 was used in the following experiments. High-frequency transducing particles/phages (HFTPs) carry a highly-expressing gene (*tac*-promoted *inaW*) encoding ice nucleation activity (INA), and were shown in Example 3 to allow the sensitive detection of *S. typhimurium* strain LT2 cells. The following set of experiments demonstrated that P22-derived TPs obeyed the known host range of P22 (since the biological specificity of the transduction assay is one of its key features), that different susceptible bacterial strains were transducible/detectable with equal efficiency, and that the presence of non-P22-susceptible bacteria in the assay samples did not interfere with the detection of susceptible bacteria. The results from two experiments are presented below: one examined transduction in pure cultures of bacteria, while the other examined transduction in mixed cultures.

A.   Pure Cultures

Several *Salmonella* strains which included 9 different serotypes belonging to serogroups B, D, C_1, C_2 and G_2 of the Kauffmann-White classification scheme for the genus *Salmonella* (Kauffmann (1978) *Das Fundament*, Munksgaard, Copenhagen), were provided by Dr. B. Stocker. The known presence or absence of the P22 receptor molecule in the outer membranes of the various strains allowed them to be grouped as P22-sensitive ($P22^s$) or P22-resistant ($P22^r$); thus, strains belonging t serogroups B and D are $P22^s$ while those belonging to ser groups C_1, C_2, and G_2 are $P22^r$. Transduction assays

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were performed on pure cultures of each bacterium to confirm that transduction would only occur in known P22[s] strains. *S. typhimurium* strain LT2 (P22[s]) was included in the panel as a control, since its behavior in transduction by the HFT system had been previously determined (see Example 3).

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        Pure cultures of each strain were grown in L-broth at 37°C to mid-exponential phase (A_{600} ranged from 0.28 to 0.67); serial dilutions of each culture were then made (using sterile LB as the diluent) in decades down to 10^{-7} relative to the original culture. Transduction using RGS12 (prepared as in Example 3) HFTPs was then performed according to the method outlined in Example 3. Small aliquots of each culture dilution (0.1 ml) were prepared, each of which received 25 ul of HFTP preparation containing 3×10^6 pfu total. The samples were incubated 1 hour at 37°C followed by 1 hour at 23°C. After incubation, a small amount (1 ul) of a concentrated fluorescent dye (used to aid in the detection of freezing events during the subsequent INA assay) was added to each transduced culture dilution as described in Example 3. Three replicate 10 ul droplets of each transduced dilution were then placed in separate wells of a 96-well microtiter dish. The microtiter dish was then placed on a form-fitted chilling block which was equilibrated at -10.0°C. After 10-15 minutes, the number of frozen drops was tallied. Results are shown in Table 7.

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TABLE 7

Strain	Sample/Dilution						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
S. gallinarium (D_1)	3	3	3	3	3	3	3
S. dublin (D_1)	3	3	3	3	3	3	3
S. bovis-morbificans (C_2)*	0	0	0	0	0	0	0
S. havana (G_2)*	0	0	0	0	0	0	0
S. enteritidis (D_1)	3	3	3	3	3	3	3
S. paratyphi-B (B)	3	3	3	3	3	3	3
S. typhimurium (B)	3	3	3	3	3	3	3
S. cholerae-suis (C1)*	0	0	0	0	0	0	0
S. typhi (D_1)	3	3	3	3	3	3	2
LT2 (B)	3	3	3	3	3	3	3

[1] Each of the 10^{-1} to 10^{-7} dilutions of *Salmonella* strains was assayed for INA following transduction with HFTPs. INA was measured at a constant temperature of -10.0°C. The data presented are numbers of drops of each sample dilution which froze, out of a total of 3 replicate drops per sample dilution. The serogroup of each strain is shown in parentheses next to the strain name; asterisks denote strains which are P22[r], the remainder are P22[s].

The results presented in Table 7 show that the transduction of INA occurred only among the P22[s] strains, and that furthermore, the susceptible bacteria were detected with equally high sensitivity in every case. Among the P22[r] strains, no background of INA was detected. Based on the A_{600} measurements of the original (undiluted) log-phase cultures, the cell populations of the corresponding 10^{-7} dilutions (and hence the minimum detection limit in this experiment) were fewer than 100 cells/ml. This level of sensitivity agreed with that observed previously with strain LT2 alone (see Example 3). This experiment thus demonstrated that P22-mediated transduction obeyed the kn wn h st range of the

phage, and that a variety of P22^s strains could be
transduced with equal, and high, efficiency.

B.   **Mixed Cultures**

Another aspect of the specificity of the
transduction assay is the effect that "non-target"
bacteria (i.e. miscellaneous bacteria not expected to be
detected by the transduction assay) might have on the
detection of P22^s bacteria by phage transduction. The
following experiment was done to examine transduction in
such a setting.

RGS12 HFTPs were used in this experiment
similarly to the method of part A of this Example except
as stated otherwise below. Only one P22^s strain,
S. dublin, was used and the samples were prepared so that
S. dublin was assayed while in the presence of a P22^r
bacterium. The P22^r bacteria were: *E. coli* strain
JC10291 (Willis et al. (1981) Mol. Gen. Genet. 183 479-
504), *B. subtilis* strain BR151 (ATCC No. 33677), and
S. havana (a naturally P22^r strain of *Salmonella*) -- see
part A of this Example. Each strain was grown in L broth
at 37°C to mid-exponential phase, at which time A_{600}
readings were made. The cultures were then adjusted (by
dilution in fresh L broth) to the same density (A_{600} =
0.36).

Control transductions of individual strains
were done to check the background (if any) in the P22^r
bacteria, and to measure the response of the assay with a
pure culture of dublin *S. dublin*. The *S. dublin* samples
were prepared by diluting the original (A_{600} = 0.36)
culture in decades down to 10^{-9}; only the 10^{-2} to 10^{-9}
dilutions were used in the assay. Four such dilution
series were prepared: in one, sterile LB was used as the
diluent; in the other three, one of each of the undiluted
(A_{600} - 0.36) cultures of P22^r bacteria was used as the
diluent. In other words, each such sample series
consisted of eight dilutions in which the concentration
of *S. dublin* ranged from 10^{-2} to 10^{-9} (relative to the

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original culture).   Since one of the other undiluted
cultures was used as the diluent for the *S. dublin*
dilution series, the other bacterium was present at
undiluted strength in each of the *S. dublin* dilutions.
5 So, each P22^r bacterium was present in a range of excess:
from 10^2-fold (in the 10^{-2} *S. dublin* dilutions) to 10^9-fold
(in the 10^{-9} *S. dublin* dilutions).

Each sample/dilution contained 0.1 ml total
volume, and each was transduced individually by adding
10 25 ul of RGS12 TPs (containing 3×10^6 pfu total).   To
accommodate the large number of individual samples, the
procedure was performed in a sterile 96-well microtiter
dish with lid.   Incubations were done for 1 h. at 37°C
followed by 1 h. at 23°C.   After incubations, a small
15 amount (1 ul) of a fluorescent dye concentrate was added
to each sample to aid in the detection of freezing events
during the subsequent INA assays as described in
Example 3.   Four replicate 10 ul drops of each sample
were then placed in separate wells of a flexible plastic
20 96-well microtiter dish which was placed on a form-fitted
chilling block equilibrated at -10.0°C.   After 10-15
minutes, frozen drops were tallied.   The results are
shown in Table 8.

TABLE 8

S. dublin Dilution Factor

Sample Series	10^{-3}	10^{-4}	10^{-5}	10^{-5}	10^{-7}	10^{-8}	10^{-9}
S. dublin (alone)	4	4	4	4	4	1	0
S. dublin + E. coli	4	4	4	4	3	1	0
S. dublin + B. subtilis	4	4	4	4	1	2	2
S. dublin + S. havana	4	4	4	4	3	4	0

[1] Each dilution of each sample was assayed for INA following transduction with HFTPs. INA was measured at a constant temperature of -10.0°C. Numbers in table indicate the number of droplets which froze for each dilution of sample series (out of a total of 4 drops per sample dilution). Numbers in column headings indicate the dilution factor of the S. dublin culture into undiluted cultures of the other bacteria (except where S. dublin was assayed alone).

The results shown in Table 8 demonstrate that the presence of other, P22^r, bacteria in the assay samples had no apparent effect on the detection of the target strain. The assay detected no background activity, either in the untransduced bacteria, or in the "transduced" (i.e. exposed to TPs) P22^r bacteria. In other words, the P22^r bacteria were invisible to the assay, and when present with a P22^s bacterium, they exerted no significant effect on the detection of the bacterium. Because the results described above in Part A of this Example (using pure cultures) failed to reach the lower detection limit of the assay (i.e., the highest dilutions still froze), the dilution series was extended in the present experiment to 10^{-9} in an attempt to reach below the detection limit. Since the original cultures contained in the neighborhood of 10^8 to 10^9 cells (according to an estimate of bacterial population based on A_{600}), few if any cells would be expected in the 10^{-9} dilutions. Thus, these results show that, even in the presence of large numbers of non-target bacteria, the limit of detecti n of this assay extended close to the

theoretical limit of any bacterial assay: the point at which no cells are present.

To summarize the above results, the response of a P22/*inaW* transduction system was tested against several bacteria which represented both P22-sensitive and P22-resistant strains. The response of the assay was assessed in terms of its predicted specificity (according to the known presence/absence of P22 receptors in individual strains), its sensitivity in the detection of susceptible strains, and its resistance to interference due to the presence of large numbers of non-susceptible bacteria. The assay proved to be strictly specific: no activity resulted in known $P22^r$ strains. Moreover, the sensitivity of the assay was equally high in all $P22^s$ strains which were assayed; in every case the assay performed at its optimal level as determined in previous experiments using strain LT2 alone. Finally, the sensitivity of the assay was shown to be unaffected by the presence of large numbers of $P22^r$ bacteria; the assay still performed at its optimal level.

6. Transduction of an Enzymatic Marker Gene

This example describes a bacterial assay based on the transduction of an enzymatic marker gene. The system used the P22-mediated transduction of a gene encoding beta-glucuronidase, or "GUS", activity; transduced, GUS^+ bacteria were then detected by a fluorescent assay.

GUS activity is encoded by the *uidA* gene which was originally isolated from *E. coli* K12 (Jefferson et al. (1986) Proc. Natl. Acad. Sci. USA 83 8447-8451). It is a well-characterized enzymatic marker system which has been shown to function in a range of organisms from bacteria to higher plants (Jefferson et al. (1987) EMBO J. 6 3901-3907). Reagents for the flu rescent assay of GUS activity are c mmercially available.

The GUS transduction system was designed using phage P22, in a manner analog us to that used to

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construct the *inaW* high-frequency transduction system
described in Example 3. In the present example, the *uidA*
gene was incorporated into a P22 prophage, located in the
chromosome of a lysogenic host bacterium, in such a way

5   that induction of the phage's lytic cycle resulted in the
replication and packaging of the *uidA* sequences along
with the phage's own DNA.

The *uidA* clone was obtained from the plasmid
PJJ3431, in which the gene had been modified slightly to

10   provide an *NcoI* site at the start codon. The
modifications introduced 5 extra amino acids to the amino
terminus of the gene product; however, GUS activity was
retained. The *uidA* gene was isolated on a 2.9 kb
NcoI/PstI restriction enzyme fragment of pJJ3431 and

15   cloned into the corresponding sites of the expression
vector pKK233-2 (Brosius, et al. (1985) J. Biol. Chem.
266 3539-3541). pKK233-2 provided the *uidA* gene with a
powerful hybrid promoter, called "*trc*", a ribosome
binding site and a start codon; additionally, it encoded

20   ampicillin resistance (Ap^r) and contained a region ("bom")
which allowed it to be mobilized by certain bacterial
conjugated systems. The resulting construct was called
pRLG73.

pRLG73 was then furnished with a region of P22

25   DNA, to provide a target for homologous recombination
which would allow the plasmid to be incorporated into a
P22 prophage during a subsequent stage of the
construction. The 2.5 kb P22 *BamHI* fragment "B" (as
designated in the P22 physical map of Rutila and

30   Jackson -- see Example 3) was cloned into the unique
BamHI site of pRLG73, just upstream of the *trc* promoter
region. Since the P22 fragment would ultimately be the
point at which the plasmid was inserted into the phage
genome, it was selected so as not to interrupt any

35   essential P22 genes. The resulting P22/pRLG73 plasmid
was called pRLG80.

The following stages of the construction were
performed exactly as the equivalent st ps outlined in
more detail for the *inaW* transduction system described in
Example 3, with pRLG80 taking the place of pRLG68.

5   pRLG80 was transferred by bacterial conjugation from an
E. coli donor strain into an *S. typhimurium* recipient
which was lysogenic for P22. Additionally, the *S.
typhimurium* recipient, called RGS11, contained a mutation
in one of its DNA polymerase genes (the mutant allele is

10 designated "*polA*") which prohibited the autonomous
replication of the pRLG80 plasmid. Hence, the only means
..of survival for pRLG80 in RGS11 was via integration into
the P22 chromosome, which occurred within the region of
homology carried on the plasmid. Such progeny cells were

15 selected following the conjugation by means of the Ap^r
marker carried by pRLG80; the pRLG80-carrying progeny
were also identified by the GUS activity which they
expressed (visible when a colorimetric substrate for GUS
was included in the plating medium).

20   The new P22/pRLG80 lysogenic strain was called
RGS34, and was the strain which was used to produce *uidA*
transducing phages (TPs). Production of TPs was
accomplished by treating a liquid culture of RGS34 with
the drug mitomycin-C, as described in Example 3.

25 Mitomycin-C treatment results in the replication and
packaging of progeny phage particles which are released
into the medium following the lysis of the host cell. In
the case of RGS34, such induction resulted in the
production of TPs as well as normal phages (which arise

30 by the excision of the plasmid DNA during the replication
cycle). However, RGS34 was found to produce an
exceptionally low number of normal phages, as measured by
a plaquing assay. TPs are expected to be defective for
plaque f rmati n, since the presence f n n-phage DNA.

35 prohibits them from carrying a full genome of phage DNA
(the phages are limited in the amount of DNA that they
can carry). Hence, a plaque-f rming assay is n t a

direct measure of the number of TPs present, but only
serves to provide a reference number to characterize the
TP preparation. The RGS34 TP preparation contained 7 x
10^4 plaque forming units (pfu) per ml. In contrast, the

5   induction of the normal P22 lysogen in the RGS11
progenitor strain would have produced about 1 x 10^8
pfu/ml.

DNA of RGS34-derived phages was prepared for
its analysis as follows: 30 ml of RGS34 mitomycin-C-

10   induced TPs were centrifuged at 72,000 g for 3 h. to
pellet phages. The phage pellet was resuspended in 0.5
ml of TE buffer (10 nM tris-HCl, 1 mM Na_2EDTA, pH 8.0);
phages were then treated with protease by the addition of
Na_2EDTA, Sodium Dodecyl-Sulfate, and Proteinase-K to final

15   concentrations of 20 mM, 0.5% and 50 ug/ml, respectively,
followed by incubation at 37°C for 1h. Following
protease treatment, three phenol extractions and three
ether extractions were done, then phage DNA was
precipitated by the addition of ethanol to 66%. The

20   purified phage DNA was dissolved in 100 ul of TE buffer.
Restriction digests of RGS34 TP DNA were analyzed by gel
electrophoresis, which showed the presence of extra bands
(relative to normal P22 DNA) that corresponded with those
expected from the incorporation of pRLG80 into the phage

25   genome. This DNA analysis confirmed that TPs were in
fact being produced by the RGS34 strain.

The RGS34 mitomycin-C-induced TP preparation
initially contained bacterial cell debris which was
pelleted by centrifugation at 4,000xg for 10 minutes

30   (while leaving TPs still in suspension). However, much
GUS activity was released during the lysis of TP-
producing host cells, which remained in the TP
suspension. Any GUS activity contained in the TP
suspension would likely obscure the activity which

35   resulted from transduction of the *uidA* gene, making it
necessary to first purify the TP preparation prior to
use. This was done by two successive rounds of

centrifugation at 48,000 g to pellet the TPs, followed by resuspension of the TPs into fresh L-broth. This effectively diluted the GUS activity of the TP prep to a level near the background of the fluorescent GUS assay, described below.

The transduction assay was done by adding RGS34 TPs to exponential-phase cultures of bacteria, incubating several hours, then making an extract of the transduced cells which was assayed for GUS activity using the fluorescent substrate method. Cultures of *S. typhimurium* strain LT2 (a P22-sensitive strain) and *S. havana* (a P22-resistant strain of *Salmonella*) were grown at 37°C to exponential phase, then adjusted (by dilution in fresh L-broth) to A_{600} = 0.21 at the start of the experiment. Samples of sterile L-broth were included to allow the measurement of contaminating GUS activity added with the TP preparation, if any. To each 0.2 ml sample was added 0.1 ml of TP prep which contained 7×10^3 pfu total; a set of control samples was prepared similarly which, instead of TPs, received an equivalent amount of sterile LB. The samples were then incubated at 37°C for 3.5 hours, after which the bacteria were pelleted by centrifugation and resuspended into 0.25 ml of GUS extraction buffer (50 mM Na-PO$_4$ pH 7.0, 1 mM Na$_2$EDTA, 0.1% Triton X-100, 10 mM B-mercaptoethanol). 5 ul of toluene was then added to each cell suspension (to permeabilize the cells allowing the release of the GUS enzyme into the extraction buffer), vortexed 40 seconds, then let stand at room temperature for 10 minutes. The samples were then centrifuged to pellet cell debris and separate phases, and 0.19 ml of each extract was removed to a fresh tube.

GUS assays were begun by adding 10 ul of a 20 mM solution of 4-methyl umbelliferyl glucuronide ("MUG" - the flu rescent substrate for GUS) t each 0.19 ml of cell extract. MUG itself is not fluorescent, but up n reaction with GUS releases the then-fluorescent 4-methyl umbelliferone group. Assays were incubated 30

minutes at 37°C, at which time 50 ul aliquots of each
assay sample were removed and added to 0.95 ml of 0.2M
Na_2CO_3 (the Na_2CO_3 stops the reaction and enhances the
fluorescence of the reaction product). Fluorescence of
the sample aliquots was then measured in a fluorescent
spectrophotometer with excitation of the samples at 365
nm; fluorescent emissions were measured at 455 nm. The
results are shown in Table 9.

TABLE 9

Sample		Fluorescence Units
1)	LT2 Control	45
2)	LT2 + TPs	1183
3)	S. havana Control	98
4)	S. havana + TPs	46
5)	L broth Control	38
6)	L broth + TPs	82

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[1] Numbers indicate the fluorescence of samples
     measured with excitation at 365 nm and emission at
     455 nm. Raw fluorescence units may be converted
     into the corresponding concentrations of 4-methyl
     umbellyferone (the fluorescent compound which is
     released by reaction of MUG with GUS), but serve
     here as relative measurements of GUS activity.

        Table 9 shows that the only strongly positive
reading of GUS activity occurred in the transduced LT2
sample (Sample 2). Since the residual GUS activity in
the TP preparation (as determined by the difference
between Samples 5 and 6) was negligible, the activity
seen in Sample 2 was due to transduction. In contrast,
the S. havana strain showed no increase in GUS activity
due to exposure to the TPs. Thus, the GUS transduction
system was shown to be operative for the P22[s] strain
(LT2), but not for the P22[r] strain (S. havana). The
background fluorescence measured in these samples was not
likely the result of any intrinsic GUS activity in these
strains (nor in sterile L-broth), but was pr bably due to

the presence of small amounts of free 4-methyl umbellyferone in the MUG preparation.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

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WHAT IS CLAIMED IS:

1.   A method for specifically detecting target
bacteria in a sample, said method comprising:
        exposing the sample to a transducing particle
specific for the target bacteria, said particle being
capable of conferring a detectable phenotype on the
bacteria; and
        detecting in the sample the presence of
bacteria possessing the detectable phenotype.

2.   A method as in claim 1, wherein the
detectable phenotype is ice nucleation activity.

3.   A method as in claim 2, wherein the
transducing particle carries an ice nucleation gene
downstream from a promoter region.

4.   A method as in claim 3, wherein the ice
nucleation gene is selected from the group consisting of
inaW, inaY, inaZ, and *iceE*.

5.   A method as in claim 1, wherein the
detectable phenotype is enzyme-catalyzed color
production.

6.   A method as in claim 5, wherein the color
production is catalyzed by β glucuronidase.

7.   A method as in claim 1, wherein the sample
is exposed to a plurality of transducing particles having
different host range specificities, whereby the bacterial
type may be determined based on the pattern of detectable
phenotype conferred by said plurality of particles.

8.   A method as in claim 1, wherein the sample
is exposed to immobilized antibodies specific for the

target bacteria prior to exposure to the transducing particle.

9.   A method as in claim 1, wherein sample is selected from the group consisting of patient specimens, water, dairy products, and meat products.

10.   A method as in claim 1, wherein the sample contains target bacteria and non-target bacteria and wherein said bacteriophage is specific for the target bacteria but not for the non-target bacteria.

11.   A method as in claim 1, wherein prior to exposure to the transducing particle, the sample has been exposed to conditions which might debilitate target bacteria.

12.   A method as in claim 10, wherein the exposure conditions include heating.

13.   A method as in claim 1, wherein the target bacteria are of the genus *Salmonella*.

14.   A method as in claim 1, wherein the transducing particle is derived from a bacteriophage selected from the group consisting of P22, L, ViI, 8, 23, 25, 31, 46, 102, 163, and 175.

15.   A method for specifically detecting target bacteria in a sample, said method comprising:
        incubating said sample in a growth medium in the presence of a transducing particle specific for the target bacteria under conditions which promote attachment of said particles to said bacteria, said transducing particle being capable of c nferring an ice nucleation phen type on the bacteria;

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subsequently incubating said sample under
conditions which promote the development of the ice
nucleation phenotype; and
detecting in the sample the presence of
bacteria possessing the ice nucleation phenotype.

16.   A method as in claim 15, wherein the
sample is first incubated at a temperature in the range
from about 35°C to 40°C without agitation for a period
from about 15 to 120 minutes.

17.   A method as in claim 16, wherein the
sample is subsequently incubated at a temperature in the
range from 20°C to 25°C for a period from about 30
minutes to 2 hours.

18.   A method as in claim 15, wherein the
sample is incubated in the presence of a plurality of
transducing particles having different host range
specificities, further comprising determining which
transducing particles result in transformation of the
bacteria to the ice nucleation phenotype, whereby the
bacterial type may be determined.

19.   A method as in claim 15, further
comprising exposing the sample to immobilized antibody
specific for the target bacteria and separating target
bacteria bound to said immobilized antibody prior to
incubating said sample.

20.   A method as in claim 13, wherein the
growth medium is selective for growth of the target
bacteria.

21.   A method as in claim 13, wherein th
sample is selected from the group c nsisting of patient

specimens, water, soil, dairy products, and meat
products.

22. A method as in claim 13, wherein the
transducing particle carries an ice nucleation gene
downstream from a promoter region.

23. A method as in claim 13, wherein the
transducing particle becomes incorporated as a prophage
within the genome of the target bacteria.

24. A method as in claim 13, wherein the
presence of bacteria possessing the ice nucleation
phenotype is detected by an ice nucleation assay.

25. A method as in claim 24, wherein the ice
nucleation assay comprises a fluorescence freezing assay.

26. A method for specifically detecting target
bacteria, said method comprising:
    transforming the target bacteria to express a
preselected cell surface receptor;
    introducing the transformed target bacteria to
an environment;
    collecting bacteria from the environment;
    exposing the collected bacteria to a
transducing particle specific for the surface receptor.
said particle being capable of conferring a detectable
phenotype on the bacteria; and
    detecting in the collected bacteria the
presence of bacteria possessing the detectable phenotype.

27. A method as in claim 26, wherein the
environment into which the transf rmed bacteria are
introduced contains other bacteria which ar
substantially the same as the target bacteria except that
they lack the cell surface receptor.

28. A method as in claim 26, wherein the target bacteria is a *Pseudomonas* species and the cell surface receptor is encoded by a *lamB* gene.

29. A method as in claim 26, wherein the environment is selected from soil, air, and water.

30. A transducing particle capable of conferring an ice nucleation phenotype on infected bacterial cells.

31. A transducing particle as in claim 30, capable of specifically infecting gram-negative bacteria.

32. A transducing particle as in claim 31, wherein the gram-negative bacteria are selected from the group consisting of *Escherichia coli, Vibrio cholerai*, and *Salmonellae*.

33. A transducing particle as in claim 30, capable of specifically infecting gram-positive bacteria.

34. A transducing particle as in claim 33, wherein the gram-positive bacteria are selected from the group consisting of *Staphylococcus aureus* and *Streptococcus pyogenes*.

35. A transducing particle as in claim 30, having an ice nucleation gene downstream from a promoter.

36. A transducing particle as in claim 35, comprising a selective marker in addition to the ice nucleation gene.

37. A transducing particle as in claim 36, wherein the additional selective marker is antibiotic resistance.

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38. A bacterial cell having a prophage including an ice nucleation gene under the transcriptional control of a promoter.

39. A bacterial cell as in claim 38, wherein
10 the ice nucleation gene is selected from the group consisting of inaW, inaY, inaZ, and iceE.

40. A bacterial cell as in claim 38, which is a strain of E. coli or S. typhimurium.

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41. A method for producing transducing particles capable of conferring an ice nucleation phenotype on infected bacterial cells, said method comprising:
20   inducing bacterial cells having a prophage including an ice nucleation gene under the transcriptional control of a promoter; and
       collecting transducing particles released as said bacterial cells are lysed.

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42. A method as in claim 41, wherein the ice nucleation gene is selected from the group consisting of inaW, inaY, inaZ, and iceE.

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43. A method for producing transducing particles capable of conferring a detectable phenotype on infected bacterial cells, said method comprising:
       combining a gene encoding the detectable phen type with the essential terminal DNA sequences of a
35 transposon to produce an insertable element; and

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introducing the insertable element into a
bacteriophage having a genome capable of recombination
with the insertable element.

5   44.   A method as in claim 43, wherein the gene
is selected from the group consisting of ice nucleation
genes and color-producing enzyme genes.

           45.   A method as in claim 43, wherein the
10   insertable element is present in a bacterial plasmid and
introeuced to the bacteriophage by recombination in a
bacterial cell.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/04388

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (4): C12Q1/70, G01N 33/53, C12Q 1/06, C12N 15/00.
U.S. Cl: 435/5, 7 , 39 , 172.3.

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S.	435/5,7,34,39,172.3,235,320,252.3,252.33,252.8

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

Chemical Abstracts Servies Online (File CA, 1967-1988; File Biosis Previews 1969-1988). Automated Patent System (File USPAT, 1975-1988).

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X / Y	US, A, 4,650,761 (HERSHBERGER) 17 March 1987, see entire document.	1 / 2-4,6,7, 10-15,21-25 and 26-45
Y	US, A, 4,464,473 (ORSER) 7 August 1984, see entire document.	2-15 and 21-45
Y	Zinsser Microbiology, Eighteenth Edition, Published 1984 (Norwalk, Connecticut, USA), W. K. JOKLIK, Appleton-Century-Crofts, a Publishing Division of Prentice-Hall, Inc., see pages 447-448.	7,11,13,14, 18,30-35 and 43
Y	Virology, volume 50, Published 1972, T. WATANABE, "Specialized Transduction of Tetracycline Resistance By Phage P22 In Salmonella typhimurium", see entire document.	13-20

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27 December 1989	1 7 JAN 1990

International Searching Authority	Signature of Authorized Officer
ISA/US	LAURIE A. SCHIETNER

Form PCT/ISA/210 (second sheet) (Rev.11-87)